Thermosyntropho lipolytica gen. nov., sp. nov., a Lipolytic, Anaerobic, Alkalitolerant, Thermophilic Bacterium Utilizing Short- and Long-Chain Fatty Acids in Syntrophic Coculture with a Methanogenic Archaeum

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Three strains of an anaerobic thermophilic organoheterotrophic lipolytic alkalitolerant bacterium, *Thermosyntropho lipolytica* gen. nov., sp. nov. (type strain JW/VS-265^T; DSM 11003), were isolated from alkaline hot springs of Lake Bogoria (Kenya). The cells were nonmotile, non-spore forming, straight or slightly curved rods. At 60°C the pH range for growth determined at 25°C [pH^{25°C}] was 7.15 to 9.5, with an optimum between 8.1 and 8.9 (pH^{60°C} of 7.6 and 8.1). At a pH^{25°C} of 8.5 the temperature range for growth was from 52 to 70°C, with an optimum between 60 and 66°C. The shortest doubling time was around 1 h. In pure culture the bacterium grew in a mineral base medium supplemented with yeast extract, tryptone, Casamino Acids, betaine, and crotonate as carbon sources, producing acetate as a major product and constitutively a lipase. During growth in the presence of olive oil, free long-chain fatty acids were accumulated in the medium but the pure culture could not utilize olive oil, triacylglycerols, short- and long-chain fatty acids, and glycerol for growth. In syntrophic coculture (*Methanobacterium* strain JW/VS-M29) the lipolytic bacteria grew on triacylglycerols and linear saturated and unsaturated fatty acids with 4 to 18 carbon atoms, but glycerol was not utilized. Fatty acids with even numbers of carbon atoms were degraded to acetate and methane, while from odd-numbered fatty acids 1 mol of propionate per mol of fatty acid was additionally formed. 16S rDNA sequence analysis identified *Syntrophospora* and *Syntrophomonas* spp. as closest phylogenetic neighbors.

Until now, thermophilic anaerobic lipolytic bacteria able to utilize long-chain fatty acids in syntrophic cocultures were unknown. This is surprising since a large number of obligately anaerobic thermophilic and extremely thermophilic organotrophic hydrolytic (eu)bacteria and archaea have been isolated from geothermally heated and anthropogenic thermobiotic environments (41). Most of these microorganisms grow at pH values close to neutral except for a few recently isolated proteolytic anaerobic alkali(tolerant)-thermophiles (8, 20, 21). Most of the known lipolytic microorganisms are aerobes, which include some recent examples of thermophiles and alkaliphiles (7, 16, 17, 36, 38). All known anaerobic lipolytic bacteria which produce true lipases, such as *Anaerovibrio lipolytica* (29) and a *Butyrivibrio* sp. (12) from rumina, are mesophilic neutrophiles (24). They are able to grow anaerobically on triacylglycerides, hydrolyzing the acylglycerol linkage and utilizing the liberated glycerol for growth, but do not utilize the long-chain fatty acids. The short- and long-chain fatty acids under anaerobic conditions are degraded by nonlipolytic syntrophic proton-reducing microorganisms (1, 2, 4, 14, 23–26, 34, 35, 37, 43, 44) and by some nonlipolytic sulfate-reducing bacteria (32, 40); however, they depend on other lipolytic organisms, such as *A. lipolytica* (29). Only two nonlipolytic, neutrophilic proton-reducing mesophiles have been described, the syntrophic *Syntrophomonas sapovorans* and *Syntrophomonas wolfei* subsp. *saponavida*, which are able to degrade long (18-carbon)-chain fatty acids via β -oxidation in syntrophic associations with H_2 -utilizing methanogens or sulfate reducers (23, 34).

To our knowledge, no lipolytic, obligately anaerobic neutrophilic or alkaliphilic thermophile from any natural volcanic or man-made thermobiotic environments nor an anaerobic thermophile which hydrolyzes triglycerides and utilizes the liberated short- and long-chain fatty acids for growth has been described. Recently, an anaerobic thermophilic (55°C) enrichment culture able to degrade a wide range of short- and long-chain fatty acids was described (1). Here, we report on the first thermophilic syntrophic anaerobic alkalitolerant lipolytic eubacterium, *Thermosyntropho lipolytica* gen. nov., sp. nov., isolated from alkaline volcanic environments of Lake Bogoria, Kenya. It presents a novel physiological type among the thermophiles. When incubated in the presence of triglycerides, this bacterium hydrolyzed triglycerides and grew on the liberated fatty acids in a syntrophic association with an alkalitolerant H_2 -utilizing methanogen, isolated from the same environment.

MATERIALS AND METHODS

Isolation and culture conditions. The prereduced basal medium used for enrichment, isolation, and cultivation of lipolytic bacteria was prepared by the modified Hungate technique (22) under nitrogen gas phase. The basal medium contained (per liter) 0.3 g of K_2HPO_4 , 0.3 g of KCl, 0.5 g of NaCl, 1.0 g of NH_4Cl , 0.3 g of $MgCl_2 \cdot 6 H_2O$, 0.05 g of $CaCl_2 \cdot 2 H_2O$, 3.0 g of $NaHCO_3$, 3.0 g of Na_2CO_3 , 0.5 g of $Na_2S \cdot 9 H_2O$, 0.15 g of cysteine, 2 ml of vitamin solution (11), and 2.5 ml of a trace element solution (11). Substrates were added in concentrations indicated below. After addition of the substrates, the pH of the medium was adjusted to values indicated below at 25°C (pH^{25°C}) or at cultivation temperature (e.g., pH^{60°C}).

For the enrichment of lipolytic bacteria, 20 ml of commercial olive oil (Kroger brand) per liter and 5 g of yeast extract per liter were added to the basal medium and the pH^{25°C} was adjusted to 8.8 and 9.5. The enrichments were incubated at 60, 70, and 80°C. Serial dilutions of positive incubations were made into the same medium (pH^{60°C} 8.0), and the mixtures were incubated at 60°C. Partially purified lipolytic cocultures obtained by serial dilutions in liquid medium were serially diluted and used to inoculate agar-shake-roll tubes containing basal medium (pH^{60°C} 7.8) with 5 ml of emulsified olive oil, 0.5 g of yeast extract, and 3 mg of rhodamine B (19) per liter and 2% agar. The agar-shake-roll tubes were incu-

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bated at 60°C for 2 to 3 days until colonies appeared. Colonies were picked, transferred to liquid medium, serially diluted and used as inocula for subsequent agar-shake-roll tubes. Several rounds of isolating single colonies were carried out to ensure the purity of the strains with 1% (wt/vol) yeast extract-containing media.

Light microscopy. Light microscopy was performed with a model PM-10AD phase-contrast microscope (Olympus Optical Co., Ltd., Tokyo, Japan).

Determination of growth. Growth of the lipolytic bacteria and the lipolytic cocultures on media containing olive oil, triglycerides, or long-chain fatty acids was determined by direct cell counting using a phase-contrast microscope and a Petroff-Hausser bacterial counter (Hausser Scientific Partnership, Horsham, Pa.). On media with soluble substrates, growth was determined by counting and by measuring the increase in optical density at 600 nm (Spectronic 21; Bausch & Lomb, Rochester, N.Y.).

pH and temperature ranges for growth. The pH range for growth was determined for growth in the basal medium containing 10 g of yeast extract per liter as the carbon source. For determination of the pH range for growth the pH value of the medium was adjusted at 60°C (pH^{60°C}) with a model 825-MP pH meter calibrated at 60°C (Fisher Scientific, Pittsburgh, Pa.) equipped with a combination pH electrode (Sensor, Staton, Calif.) and a Fisher Scientific temperature probe. The pH values were also determined at 25°C (pH^{25°C}) to allow comparison of values from the literature (e.g., pH^{60°C} values of 6.85, 7.5, 8.2, and 9.0 correspond to pH^{25°C} values of 6.9, 8.0, 9.0, and 10.0, respectively). For the determination of the temperature range for growth the cultures were incubated at different temperatures under shaking (Temperature Gradient Incubator; 15 shakes per minute; Scientific Industries, Inc., Bohemia, N.Y.) at pH^{25°C} 8.5 (pH^{60°C} 7.8) with growth determination by the direct counting method.

Substrate utilization. Autoclaved (oil, triglycerides, and long-chain fatty acids) or filter-sterilized substrates were added to the basal medium containing 2 g of yeast extract per liter (pH^{25°C} adjusted to 8.5). The cultures were incubated at 60°C for 2 weeks, and substrate utilization was determined by periodical determination of cell counts, pH, and fermentation products, including hydrogen and methane, in the syntrophic cultures. Medium containing only 2 g of yeast extract per liter was used as control.

Fermentation products. Determination of short-chain fatty acids and alcohols was performed by high-performance liquid chromatography (HPLC) using an Aminex HPX-87 column (300 by 7.5 mm) (Bio-Rad, Richmond, Calif.) (eluent, 40 mM H₂SO₄; column temperature, 65°C; flow rate, 0.6 ml/min) and a Refractive Index Detector 156 (Altax, San Ramon, Calif.) at 35°C. The injection volume was 100 µl. Methane and hydrogen were determined by gas chromatography (chromatograph AGS 111 HS with a thermal conductivity detector from Carle, Loveland, Colo.) using N₂ as the carrier gas for H₂ determination and He for the CH₄ determination.

Determination of long-chain fatty acids. The concentrations of long-chain fatty acids in cultures grown in the presence of triacylglycerides and in lipase activity assays were measured enzymatically with an assay kit. The kit is based on the reaction of acyl-coenzyme A synthetase and acyl-coenzyme A oxidase (NEFA-Kit-U, NESCAUTO).

Lipase assay. The lipase activity of culture supernatants was determined for 2 to 16 h on a shaker at 50°C with olive oil or *para*-nitrophenyl laurate (pNP-laurate) as the substrate. When olive oil was used, the reaction mixture contained 1.3 ml of olive oil emulsion (final concentration, 5%, [vol/vol]) in 100 mM 3-[*N*-tris(hydroxymethyl)methylamine]propanesulfonic acid (TAPS) (pH^{50°C} 9.0) or 100 mM 3-cyclohexylamino-1-propanesulfonic acid (CAPS) (pH^{50°C} 10.0) buffer and 0.2 ml of culture supernatant. The free fatty acids liberated from olive oil were determined with the NEFA-Kit-U. When pNP-laurate was used as the substrate, the reaction mixture contained 100 µl of culture supernatant, 1,088 µl of TAPS or CAPS buffer, and 12 µl of 300 mM pNP-laurate in acetonitrile (final concentration in the assay, 3 mM). After 15 to 60 min of incubation at 50°C the reaction mixture was chilled on ice to stop the reaction and the A₄₀₅ of the centrifuged supernatant was measured. One unit was defined as the amount of the enzyme catalyzing the release of 1 µmol of *p*-nitrophenol per min from pNP-laurate at 50°C with the molar absorption coefficient of 18,200 M⁻¹ cm⁻¹.

Susceptibility to antibiotics. Susceptibility to antibiotics was determined at 60°C and pH^{60°C} 7.75 by transferring an exponentially growing culture into fresh medium containing 10 g of yeast extract per liter and 50 µg of filter-sterilized O₂-free antibiotic per ml.

Gram-staining reaction. The Gram-staining reaction was determined by the modified Hucker method (Enhanced GramStain kit; Carr Scarborough Microbiologicals, Inc., Decatur, Ga.). Gram type was determined according to Wiegand and Quandt (42).

DNA isolation and determination of G+C content. DNA was isolated by the NaOH method of Mesbah et al. (27). The guanine-plus-cytosine content (moles percent G+C) was determined photometrically after enzymatic digestion and separation by HPLC of nucleosides as described by Whitman et al. (39) and Mesbah et al. (27).

Genes coding for 16S rDNA sequence analysis. Genomic DNA was isolated from the strains JW/VS-264, JW/VS-265^T, and JW/VS-269, and the 16S ribosomal DNA was amplified as described previously (30, 31). PCR products were sequenced by using the Taq Dye-Deoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, Calif.) as recommended by the manufacturer. Purified sequence reaction products were electrophoresed by using an Applied

Biosystem model 373A DNA sequencer. The 16S rDNA sequences of the three strains were manually aligned with previously published 16S rRNA/rDNA sequences of representatives of the clostridia and related taxa. The method of Jukes and Cantor (18) was used to calculate evolutionary distances from which a phylogenetic dendrogram was reconstructed according to the algorithm of De Soete (6).

Nucleotide sequence accession number. The 16S rDNA sequence of strain JW/VS-265^T has been deposited in the EMBL database under the accession no. X99980.

RESULTS AND DISCUSSION

Isolation of anaerobic lipolytic thermophiles. Over 80 samples taken from various volcanic areas in New Zealand, the United States, Kenya, the Far East of Russia, and different sewage plants in the United States were used as inocula for enrichments of anaerobic lipolytic thermophiles. A phosphate-based mineral medium (pH^{25°C} 8.8 or 9.5, pH^{60°C} 8.0 and 8.6) containing 20 ml of commercial olive oil per liter as the carbon and energy source and 0.5 g of yeast extract per liter was employed. Stable lipolytic enrichments were obtained from three samples from alkaline hot springs on the shores of the alkaline soda-containing Lake Bogoria (Kenya) and were incubated at pH^{25°C} 8.8 (pH^{60°C} 8.0) at 60°C. The growth on olive oil was accompanied by a strong decrease of pH^{25°C} down to 7.5 and methane formation. After several transfers and serial dilutions, mainly two morphologically distinct types of microorganisms remained in these enrichments: slightly curved rods (later identified as the lipolytic bacterium) and long, more or less filamentous cells identified by epifluorescence microscopy as methanogens.

The lipolytic bacteria were isolated by serial dilutions and picking single colonies in agar-shake-roll tubes using medium supplemented with emulsified commercial olive oil (rendering the medium turbid) and rhodamine B for visualization of lipase activity. After about 1 week, incubations at 60°C yielded small irregular white colonies with clearing zones. When rhodamine B was present, the colonies with clearing zones formed orange fluorescent zones when irradiated with UV light at 350 nm, a specific test for lipases (19). All positive enrichments yielded morphologically similar types of colonies and bacteria. Three isolates with the highest lipase activity, strains JW/VS-264, JW/VS-265^T, JW/VS-269, were selected for further studies.

Colony and cell morphology. Colonies in agar (2%)-shake-roll tubes (mineral medium, 0.01% [wt/vol] yeast extract; 0.5% [vol/vol] olive oil; pH 8.5, 60°C) which appeared after 2 days of incubation at 60°C were small (diameter 0.5 mm), white, and lens shaped. Cells of the three strains were morphologically similar, straight or slightly curved rods 0.3 to 0.4 µm in diameter and 2 to 3.5 µm in length (Fig. 1). Chains of two and more cells were seen during the exponential growth phase. Motility of the cells was never observed, nor was the formation of heat-stable spores after growth under various culture conditions which included low and high substrate concentrations in liquid and solid medium, different temperatures (37 to 70°C) and pH values (pH 7.0 to 9.0), and several weeks of prolonged incubations.

Cell wall, Gram-staining reaction, and Gram type. The cells of the strain JW/VS-265^T stained gram negative in both exponential and stationary growth phases, but the organism is a gram-type-positive bacterium (42). This is consistent with the 16S rDNA sequencing data which placed the organism into the *Clostridium-Bacillus* subphylum.

Physiological characterization. (i) **Temperature and pH ranges for growth.** The temperature range for growth of the three purified strains was 52 to 70°C with an optimum at 60 to 66°C (Fig. 2A). Growth was not observed within several weeks

FIG. 1. Light microscopy of exponentially growing culture of *T. lipolytica* JW/SV-265^T during growth in mineral medium (60°C, pH^{60°C} 8.6) supplemented with 1% (wt/vol) yeast extract.

at temperatures of 44°C and below or at 73°C and above. The pH optimum for growth was pH^{25°C} 8.1 to 8.9 (pH^{60°C} 7.6 to 8.0) for strains JW/SV-264 and JW/SV-265^T and 8.1 to 8.5 (pH^{60°C} 7.6 to 7.8) for strain JW/SV-269. The strains grew slowly at pH^{25°C} 7.15 and 9.5 but not at pH^{25°C} 6.9 or 9.7 (Fig. 2B). With mineral medium supplemented with yeast extract (1%, vol/vol), the doubling time under the above optimum growth conditions was about 1 h (Fig. 2), whereas in the presence of only 0.2% yeast extract and 0.5% (vol/vol) olive oil the doubling time was 3.4 h.

(ii) **Substrate utilization by the lipolytic monocultures at 60°C.** The three strains grew well as monocultures on liquid medium with yeast extract as the sole source of carbon and energy. The cell count increased proportionally with the yeast extract concentration (0 to 1%, wt/vol) (data not shown) and reached approximately 1.5×10^7 and 1.4×10^8 cells/ml with 1.0 and 10 g of yeast extract per liter, respectively. At 60°C, strain JW/SV-265^T utilized the following substrates when added to the basal mineral medium (pH^{25°C} 8.8) containing 2 g of yeast extract per liter: maximal optical density (beyond the base value in the control without supplements) was reached within 1 to 2 days with 5 g (each tested separately) of tryptone, Casamino Acids, beef extract, brain heart infusion, or nutrient broth per liter. More than 7 days of incubation were required for growth on crotonate (15 mM), producing acetate and butyrate, and on betaine (10 mM), producing acetate. The cell count on crotonate was 8×10^7 cells/ml. With pyruvate (15 mM), ribose (2.5 g/liter), and xylose (2.5 g/liter), weak growth with concomitant formation of small amounts of acetate acidifying the medium containing sugars was seen.

In the absence of a methanogen, the addition of olive oil (0.5 to 10 ml/liter), soybean oil (0.5 to 10 ml/liter), tributyrin (3 mM), trilaurin (1 mM), tripalmitin (1 mM), tristearin (1 mM), and triolein (1 mM) did not stimulate but also did not inhibit the initial growth in basal medium containing 2 g of yeast extract per liter. The triacyl glycerides (see below for details), however, were hydrolyzed by a constitutively formed lipase found in the culture supernatant, but the liberated glycerol and long-chain fatty acids were not utilized regardless of whether CaCl₂ was added (in equimolar concentrations relative to the fatty acid content of the triacyl glycerides [3 mM]) (33). Furthermore, no growth and no accumulation of any fermentation products were observed with the 2-g/liter yeast extract-containing basal medium supplemented with (tested separately) 6.8 to 68 mM glycerol; 5 mM acetate, propionate, butyrate, isobu-

tyrate, hydroxybutyrate, valerate, isovalerate, or caproate; 2 mM heptanoate, caprylate, pelargonate, or laurate; 1 mM myristate, palmitate, stearate, oleate, linoleate, arachidate, behenate, or lignocerate; 15 mM lactate, malate, fumarate, succinate, methanol, ethanol, 1-propanol, 1-butanol(2,5); 5 g of arabinose, fructose, galactose, glucose, mannose, raffinose, sucrose, starch, xylan, or pectin per liter; 0.5 mM 1,3-benzenediol, benzoate, 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, hydroquinone, *m*-hydroxybenzoic acid, *p*-hydroxybenzoic acid, 2-hydroxybenzoic acid, or phenol; and a mixture of 80% H₂ and 20% CO₂ (vol/vol).

Growth properties of the lipolytic strain JW/SV-265^T in reconstituted syntrophic cocultures with the methanogenic strain JW/SV-M29 (similar to strains of *Methanobacterium thermoautotrophicum* [synonym, *M. thermoacaliphilum*] [3]). The main property of *T. lipolyticus* to grow on long-chain fatty acids was observed only in coculture with hydrogen-utilizing microorganisms. In base medium supplemented with olive oil the reconstituted cocultures with either one of the isolated lipolytic strains together with the isolated methanogen behaved similarly to the partially purified lipolytic enrichments containing lipolytic and methanogenic organisms.

Using the same set of substrates and conditions as for strain JW/SV-265^T, except that the medium was inoculated with 0.1 ml each of the exponentially growing cultures of strain JW/SV-265^T (grown with yeast extract as the sole carbon source) and of the methanogen strain JW/SV-M29 (grown on H₂-CO₂), the

FIG. 2. Growth of *T. lipolytica* JW/SV-265^T (●), JW/SV-265 (△), and JW/SV-269 (□) in mineral medium supplemented with 10 g of yeast extract per liter at various temperatures at pH^{25°C} 8.5 (A) and at various pH^{25°C} values at 60°C.

reconstituted coculture grew and utilized olive oil (1 to 10 ml/liter of medium) and the tested triacyl glycerides (tributyrin [3 mM] and trilaurin, tripalmitin, tristearin, and triolein [all at 1 mM]) but required the presence of CaCl_2 in equimolar concentrations relative to the fatty acyl residues in the triglycerides (33). The best growth was observed on olive oil and triolein. Acetate (leading to an acidification), methane, and glycerol were produced stoichiometrically. The coculture grew on the saturated and unsaturated fatty acids butyrate, valerate, and caproate (all at 5 mM, but only very slowly; detectable growth required 2 to 3 weeks of incubation); heptanoate, caprylate, pelargonate, caprate, and laurate (all at 2 mM); and myristate, palmitate, stearate, oleate, and linoleate (all at 1 mM) when equimolar concentrations of CaCl_2 were provided. Fatty acids with even numbers of carbon atoms were degraded to acetate and methane, while odd-numbered fatty acids yielded additionally one propionate per fatty acid degraded. The coculture did not grow and did not utilize acetate, propionate, hydroxybutyrate, isobutyrate, isovalerate and the long-chain fatty acids arachidate ($\text{C}_{20:0}$), behenate ($\text{C}_{22:0}$), and lignocerate ($\text{C}_{24:0}$). Crotonate, betaine, pyruvate, and yeast extract, substrates which supported the growth of the lipolytic monoculture, were utilized in the coculture but without an increase in cell numbers of the methanogens (count of fluorescent cells) and without methane production, indicating that no significant amounts of H_2 were produced from these substrates. Similarly to the purified lipolytic culture, glycerol, lactate, malate, fumarate, succinate, alcohols, and aromatic compounds were not utilized (absence of increase in cells and acetate formation) by the coculture.

When incubated with olive oil or oleate (1 mM), strain JW/VS-265^T alone or in coculture with the methanogenic strain JW/VS-M29 did not utilize sulfate, nitrate, or fumarate (all tested at 15 mM) as electron acceptors. Addition of NaCl was not required for growth. The addition of 5 g of NaCl per liter did not affect growth, 10 g of NaCl per liter was slightly inhibitory, and 20 g/liter totally inhibited the growth when cultured in the basal medium containing 2 g of yeast extract per liter. The growth of strain JW/VS-265^T on yeast extract was not inhibited by 100% H_2 in the gas phase. When the strain was incubated without the methanogen in the presence of olive oil, triacyl glycerides, or long-chain fatty acids, small amounts of H_2 were produced, but growth was inhibited presumably by the accumulation of the long-chain fatty acids.

Sensitivity to antibiotics. Growth of strain JW/VS-265^T was inhibited when the strain was incubated at 60°C and pH^{25°C} 8.4 in medium containing 10 g of yeast extract per liter and the following antibiotics at 50 µg/ml: ampicillin, chloramphenicol, kanamycin, neomycin, rifampin, and vancomycin. The inhibition spectrum is in agreement with that for organisms belonging to the (eu)bacteria.

DNA base composition and 16S rDNA sequence analysis. The G+C content of the DNA was between 43 and 44 mol%. 16S rDNA sequence analysis of strains JW/VS-264, JW/VS-265^T, and JW/VS-269 placed them into the *Clostridium*-*Bacillus* subphylum. The three strains were indistinguishable at the 16S rDNA level. The 16S rDNA sequence of strain JW/VS-265^T had 93% similarity to those of *Syntrophomonas wolfei* (25, 26) and *Syntrophospora bryantii* (37, 44) as the closest related species. The new isolates are clearly separated from all other anaerobic thermophiles and thermoalkaliphiles for which sequence analyses were available (Fig. 3). On the basis of 16S rDNA sequence data (as well as morphological and physiological characteristics) the strains JW/VS-264, JW/VS-265^T, and JW/VS-269 were indistinguishable but were isolated from dif-

FIG. 3. Phylogenetic dendrogram showing the positions of *T. lipolytica* JW/SV-265^T within the radiation of the two genera *Syntrophomonas* and *Syntrophospora* of the family *Syntrophomonadaceae*. Bar, 10 nucleotide substitutions per 100 nucleotides.

ferent soil samples (although from the same habitat) and, thus, represent different strains from the same species.

Differentiation of *T. lipolytica* from other thermophilic anaerobes and mesophilic syntrophic fatty acid degraders. To our knowledge, the isolated strains are the first obligately anaerobic thermophilic lipolytic bacteria, hydrolyzing triglycerides and utilizing the liberated short- and long-chain fatty acids in a syntrophic association with an H_2 -utilizing methanogen but not utilizing the liberated glycerol. Until now, no thermophilic anaerobe responsible for the degradation of triglycerides in volcanic hydrothermal biotopes had been identified. The complete anaerobic degradation of triacylglycerols and short- and long-chain fatty acids in anaerobic zones of volcanic environments can be performed by syntrophic association of the here-described novel lipolytic syntrophic bacteria and H_2 -consuming thermophilic methanogens (e.g., *M. thermoautotrophicum* [synonym, *M. alkaliphilum*]) which were always present in our lipolytic enrichments. Thus, *T. lipolytica* represents a new physiological group of thermophilic anaerobes, combining features of mesophilic anaerobic lipolytic bacteria and syntrophic long-chain fatty acid-fermenting bacteria. *T. lipolytica* differs from all other anaerobic biopolymer-degrading thermophiles (40, 41), including the recently isolated glycolytic alkalithermophiles (8, 9, 20, 21), in that it cannot utilize cellulose, starch, pectin, and xylan. It differs from long-chain fatty acid-utilizing sulfate- or sulfur-reducing bacteria because it can hydrolyze triglycerides (32, 40). But unlike the mesophilic, neutrophilic, lipolytic *A. lipolytica* (13, 15, 29) (Table 1), the newly

TABLE 1. Characteristics that differentiate *T. lipolytica* from other, phylogenetically and physiologically related organisms

Characteristic	<i>Thermosyntropha lipolytica</i> ^a	<i>Syntrophospora bryantii</i> ^b	<i>Syntrophomonas wolfei</i> subsp. <i>wolfei</i> ^b	<i>Syntrophomonas wolfei</i> subsp. <i>saponavida</i> ^b	<i>Syntrophomonas sapovorans</i> ^b	<i>Syntrophobacter wolinii</i> ^b	<i>Anaerovibrio lipolytica</i> ^b
G+C content (mol%)	43.6	37.6					
pH range	7.15–9.5	6.5–7.5			6.3–8.1		
Optimum pH	8.1–8.9				7.3		
Optimum temp (°C)	60–66	28–34			35		38
Maximum temp (°C)	70	34	30–37	30–37	45		<50
Spore formation	–	+	–	–	–		–
Lipolytic	+	–	–	–	–	–	+
Substrates used							
In pure culture							
Yeast extract	+	–					
Tryptone	+		–	–			
Casamino Acids	+						
Crotonate	+	+	+	+	–		
Betaine	+						
Pyruvate	+	–	–		–		
Glucose	–	–	–	–	–		
Ribose	+	–					+
Xylose	+						
Glycerol	–	–		–	–		+
H ₂ -CO ₂	–				+		
In coculture							
Olive oil	+						+
Tributyrin	+				–		+
Trilaurin	+						
Tripalmitin	+			–	–		
Tristearin	+						
Triolein	+						
Glycerol	–	–					+
Acetate C _{2:0}	–	–			–		
Propionate C _{3:0}	–	–	–	–	–	+	
Butyrate C _{4:0}	+	+	+	+	+		–
Valerate C _{5:0}	+	+	+	+	+		
Caproate C _{6:0}	+	+	+	+	+		
Heptanoate C _{7:0}	+	+	+	+	+		
Caprylate C _{8:0}	+	+	+	+	+		
Pelargonate C _{9:0}	+	+		+			
Caprate C _{10:0}	+	+		+	+		
Laurate C _{12:0}	+	–		+	+		
Myristate C _{14:0}	+						
Palmitate C _{16:0}	+						
Stearate C _{18:0}	+						
Oleate C _{18:1}	+						
Linoleate C _{18:2}	+	–		–	+		

^a This study.^b Values taken from references 2, 3, 13, 23, 26, 29, 34, 35, 37, and 44.^c In pure culture.

isolated strains are alkali-tolerant (pH^{25°C} range for growth, <8.0 to 9.5) thermophiles (52 to 70°C; optimum, around 8.5) (41) and they do not ferment glycerol either in pure culture or in coculture with an H₂-utilizing methanogen.

The 16S rDNA sequence analysis places the new strains clearly in the gram-type-positive subphylum and thus separates them from the gram-type-negative *Syntrophus* and related bacteria. The lipolytic nature and the syntrophic fatty acid degradation separate the novel isolates from other known thermophilic anaerobes, including our recently isolated thermoalkaliphiles (8, 9, 22, 23). The nearest neighbors in the 16S rDNA sequence-based tree (Fig. 3), the gram-positive syntrophic *Syntrophospora* and *Syntrophomonas* spp. and the homoacetogenic bacteria *Moorella thermoacetica* and *Moorella thermo-*

autotrophicum (5), differ in several important aspects (Table 1). *Syntrophobacter wolinii* (4) is a propionate-utilizing syntrophic bacterium, and *Syntrophus buswellii* (28) utilizes syntrophically benzoate. The long-chain fatty acid-utilizing syntrophic anaerobe *Syntrophomonas sapovorans* (34) is not lipolytic. Other differences between these organisms are summarized in Table 1. On the basis of physiological properties and 16S rDNA analysis, the organism described here constitutes a novel taxon that is most closely related to the mesophilic, neutrophilic syntrophic bacteria.

Description of the genus *Thermosyntropha* gen. nov. *Thermosyntropha* (Ther.mo.syn.tro'pha. Gr. adj. *thermos*, hot; Gr. prefix *syn*, with, together; Gr. verb. *trophein*, to eat; Gr. masc. n. *syntrophos*, foster brother or sister; M.L. fem. n. *thermosyntro-*

pha ["foster sisters liking it hot," referring to the fact that the bacterium grows at elevated temperatures on fatty acids only in syntrophic cultures with hydrogen-utilizing microorganisms]). The Gram reaction-negative and gram-type-positive cells are generally rod shaped, obligately anaerobic, heterotrophic, and lipolytic and grow at elevated temperatures and moderately alkaline pH values. Thermodynamically unfavorable substrates leading to hydrogen formation are utilized in syntrophic associations with H₂-consuming microorganisms. The DNA G+C content is 43 to 44 mol%. The lipolytic, fatty acid-degrading *T. lipolytica* is the type and, so far, only species in this genus. On the basis of the 16S rDNA sequence the genus *Thermosyntropho* belongs to the family *Syntrophomonadaceae* (45).

Description of *Thermosyntropho lipolytica* sp. nov. *Thermosyntropho lipolytica* (li.po.ly'ti.ca. Gr. n. *lipos*, fat; Gr. adj. *lytikos*, able to loosen; M.L. adj. *lipolytica*, referring to the property of being able to hydrolyze lipids from the formation of fatty acids and glycerol). The cells are straight or slightly curved rods, 0.3 to 0.4 by 2.0 to 3.5 µm. Most cells occur in the exponential growth phase in pairs or chains. Motility or spores have not been observed. The organism is a peptidolytic and lipolytic chemoorganotroph. Oleate, linoleate, and the saturated fatty acids butyrate (only slowly metabolized) up to stearate are β-oxidized in syntrophic association with an H₂-utilizing microorganism. All *T. lipolytica* isolates are alkalitolerant thermophiles growing between 52 and 70°C (optimum, 60 to 66°C) and in a pH^{25°C} range from 7.15 to 9.5 (optimum, 8.1 to 8.9). The DNA base composition is 43 to 44 mol% G+C.

Strain JW/VS-265^T (DSM 11003) is the type strain of *T. lipolytica*. It has a G+C content of 44 mol%.

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REFERENCES

- Angelidaki, I., and B. K. Ahring. 1995. Establishment and characterization of an anaerobic thermophilic (55°C) enrichment culture degrading long-chain fatty acids. *Appl. Environ. Microbiol.* **61**:2442-2445.
- Beaty, P. S., and M. J. McInerney. 1987. Growth of *Syntrophomonas wolfei* in pure culture on crotonate. *Arch. Microbiol.* **147**:389-393.
- Blotevogel, K.-H., U. Fischer, M. Mocha, and S. Jannsen. 1985. *Methanobacterium thermoalkalophilum* spec. nov., a new moderately alkaliphilic and thermophilic autotrophic methanogen. *Arch. Microbiol.* **142**:211-217.
- Boone, D. R., and M. P. Bryant. 1980. Propionate-degrading bacterium *Syntrophobacter wolfeii* sp. nov., gen. nov., from methanogenic ecosystems. *Appl. Environ. Microbiol.* **40**:626-632.
- Collins, M. D., P. A. Lawson, A. Willems, J. J. Cordoba, J. Fernandez-Garayzabal, P. Garcia, J. Cai, H. Hippe, and J. A. E. Farrow. 1994. The phylogeny of the genus *Clostridium*: proposal of five new genera and eleven new species combinations. *Int. J. Syst. Bacteriol.* **44**:812-826.
- De Soete, G. 1983. A least squares algorithm for fitting additive trees to proximity data. *Psychometrika* **48**:621-626.
- Emanuilova, E., M. Kambourova, M. Dekovska, and R. Manolov. 1993. Thermoalkalophilic lipase-producing *Bacillus* selected by continuous cultivation. *FEMS Microbiol. Lett.* **108**:247-250.
- Engle, M., Y. Li, C. Woese, and J. Wiegell. 1995. Isolation and characterization of a novel alkalitolerant thermophile, *Anaerobranca horikoshii* gen. nov., sp. nov. *Int. J. Syst. Bacteriol.* **45**:454-461.
- Engle, M., Y. Li, F. Rainey, S. DeBlois, V. Mai, A. Reichert, F. Mayer, P. Messmer, and J. Wiegell. 1996. *Thermobrachium celere*, gen. nov., sp. nov., a rapidly growing thermophilic, alkalitolerant, and proteolytic obligate anaerobe. *Int. J. Syst. Bacteriol.* **46**:1025-1033.
- Frasca, J. M., and V. R. Parks. 1965. A routine technique for double-staining ultrathin sections using uranyl and lead salts. *J. Cell Biol.* **25**:157-161.
- Freier, D., C. P. Mothershed, and J. Wiegell. 1988. Characterization of *Clostridium thermocellum* JW-20. *Appl. Environ. Microbiol.* **54**:204-211.
- Hazlewood, G., and R. M. C. Dawson. 1979. Characteristics of a lipolytic and fatty acid-requiring *Butyrivibrio* sp. isolated from the ovine rumen. *J. Gen. Microbiol.* **112**:15-27.
- Henderson, C. 1971. A study of the lipase produced by *Anaerobrio lipolytica*, a rumen bacterium. *J. Gen. Microbiol.* **65**:81-89.
- Henson, J. M., and P. H. Smith. 1985. Isolation of a butyrate-utilizing bacterium in a coculture with *Methanobacterium thermoautotrophicum* from a thermophilic digester. *Appl. Environ. Microbiol.* **49**:1461-1466.
- Hobson, P. N., and S. O. Mann. 1961. The isolation of glycerol-fermenting and lipolytic bacteria from the rumen of the sheep. *J. Gen. Microbiol.* **25**:227-240.
- Horikoshi, K. 1990. Microorganisms in alkaline environments. Kodansha, Tokyo.
- Jaeger, K.-E., S. Ransac, B. W. Dijkstra, C. Colson, M. van Heuvel, and O. Misset. 1994. Bacterial lipases. *FEMS Microbiol. Rev.* **15**:29-63.
- Jukes, T. H., and C. R. Cantor. 1969. Evolution of protein molecules, p. 21-132. In H. N. Munro (ed.), *Mammalian protein metabolism*. Academic Press, Inc., New York.
- Kouker, G., and K.-E. Jaeger. 1987. Specific and sensitive plate assay for bacterial lipases. *Appl. Environ. Microbiol.* **53**:211-213.
- Li, Y., M. Engle, N. Weiss, L. Mandelco, and J. Wiegell. 1994. *Clostridium thermoalkalophilum* sp. nov., an anaerobic and thermotolerant facultative alkaliphile. *Int. J. Syst. Bacteriol.* **44**:111-118.
- Li, Y., L. Mandelco, and J. Wiegell. 1993. Isolation and characterization of a moderately thermophilic anaerobic alkaliphile, *Clostridium paradoxum* sp. nov. *Int. J. Syst. Bacteriol.* **43**:450-460.
- Ljungdahl, L., and J. Wiegell. 1987. Anaerobic fermentations, p. 84-96. In A. L. Demain and N. A. Solomon (ed.), *Manual of industrial microbiology*. American Society for Microbiology, Washington, D.C.
- Lorowitz, W. H., H. Zhao, and M. P. Bryant. 1989. *Syntrophomonas wolfei* subsp. *saponavida* subsp. nov., a long-chain fatty-acid-degrading, anaerobic, syntrophic bacterium; *Syntrophomonas wolfei* subsp. *wolfei* subsp. nov.; and emended descriptions of the genus and species. *Int. J. Syst. Bacteriol.* **39**:122-126.
- McInerney, M. J. 1988. Anaerobic hydrolysis and fermentation of fats and proteins, p. 373-415. In A. J. B. Zehnder (ed.), *Biology of anaerobic microorganisms*. John Wiley & Sons, New York.
- McInerney, M. J. 1992. The genus *Syntrophomonas* and other syntrophic anaerobes, p. 2048. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), *The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, application*, 2nd ed. Springer-Verlag, New York.
- McInerney, M. J., M. P. Bryant, R. B. Hespell, and J. W. Costerton. 1981. *Syntrophomonas wolfei* gen. nov. sp. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. *Appl. Environ. Microbiol.* **41**:1029-1039.
- Mesbah, M., U. Premachandran, and W. B. Whitman. 1989. Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int. J. Syst. Bacteriol.* **39**:159-167.
- Mountfort, D. O., W. J. Brulla, L. R. Krumholz, and M. P. Bryant. 1984. *Syntrophus buswellii* gen. nov., sp. nov.: a benzoate catabolizer from methanogenic ecosystems. *Int. J. Syst. Bacteriol.* **34**:216-217.
- Prins, R. A., A. Lankhorst, P. van der Meer, and C. J. Van Nevel. 1975. Some characteristics of *Anaerobrio lipolytica*, a rumen lipolytic organism. *Antonie Leeuwenhoek* **41**:1-11.
- Rainey, F. A., M. Dorsch, H. W. Morgan, and E. Stackebrandt. 1992. 16S rDNA analysis of *Spirochaeta thermophila*: its phylogenetic position and implications for the systematics of the order Spirochaetales. *Syst. Appl. Microbiol.* **15**:197-202.
- Rainey, F. A., N. L. Ward, H. W. Morgan, R. Toalster, and E. Stackebrandt. 1993. Phylogenetic analysis of anaerobic thermophilic bacteria: aid for their reclassification. *J. Bacteriol.* **175**:4772-4779.
- Rees, G. N., G. S. Grassia, A. J. Sheehy, P. P. Dwivedi, and B. K. C. Patel. 1995. *Desulfacinum infernum* gen. nov., sp. nov., a thermophilic sulfate-reducing bacterium from a petroleum reservoir. *Int. J. Syst. Bacteriol.* **45**:85-89.
- Roy, F., G. Albagnac, and E. Samain. 1985. Influence of calcium addition on growth of highly purified syntrophic cultures degrading long-chain fatty acids. *Appl. Environ. Microbiol.* **49**:702-705.
- Roy, F., E. Samain, H. C. Dubourguier, and G. Albagnac. 1986. *Syntrophomonas saporovans* sp. nov., a new obligately proton reducing anaerobe oxidizing saturated and unsaturated long chain fatty acids. *Arch. Microbiol.* **145**:142-147.
- Schink, B. 1992. Syntrophism among prokaryotes, p. 276-299. In A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), *The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, application*, 2nd ed. Springer-Verlag, New York.
- Sigurðsladottir, S., M. Konradslottir, A. Jonsson, J. Kristjánsson, and E. Matthiasson. 1993. Lipase activity of thermophilic bacteria from Icelandic hot springs. *Biotechnol. Lett.* **15**:361-366.
- Stieb, M., and B. Schink. 1985. Anaerobic oxidation of fatty acids by *Clostridium bryantii* sp. nov., a spore forming, obligately syntrophic bacterium. *Arch. Microbiol.* **140**:387-390.
- Sugihara, A., M. Ueshima, Y. Shimada, S. Tsunasawa, and Y. Tominaga.

1992. Purification and characterization of a novel thermostable lipase from *Pseudomonas cepacia*. *J. Biochem.* **112**:598–603.
39. **Whitman, W. B., J. Shieh, S. Sohn, D. S. Caras, and U. Premachandran.** 1986. Isolation and characterization of 22 mesophilic methanococci. *Syst. Appl. Microbiol.* **7**:235–240.
40. **Widdel, F., and T. A. Hansen.** 1992. The dissimilatory sulfate- and sulfur-reducing bacteria, p. 583–624. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), *The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, application*, 2nd ed. Springer-Verlag, New York.
41. **Wiegel, J.** 1992. The obligate anaerobic thermophilic bacteria, p. 105–184. *In* J. K. Kristjansson (ed.), *Thermophilic bacteria*. CRC Press, Boca Raton, Fla.
42. **Wiegel, J., and L. Quandt.** 1982. Determination of the Gram type using the reaction between polymyxin B and lipopolysaccharides of the outer cell wall of whole bacteria. *J. Gen. Microbiol.* **128**:2261–2270.
43. **Wu, W.-M., M. K. Jain, and J. G. Zeikus.** 1994. Anaerobic degradation of normal- and branched-chain fatty acids with four or more carbons to methane by a syntrophic methanogenic triculture. *Appl. Environ. Microbiol.* **60**:2220–2226.
44. **Zhao, H., D. Yang, C. R. Woese, and M. P. Bryant.** 1990. Assignment of *Clostridium bryantii* to *Syntrophospora bryantii* gen. nov., comb. nov. on the basis of a 16S rRNA sequence analysis of its crotonate-grown pure culture. *Int. J. Syst. Bacteriol.* **40**:40–44.
45. **Zhao, H., D. Yang, C. R. Woese, and M. P. Bryant.** 1993. Assignment of fatty acid- β -oxidizing syntrophic bacteria to *Syntrophomonadaceae* fam. nov. on the basis of 16S rRNA sequence analyses. *Int. J. Syst. Bacteriol.* **43**:278–286.